COMPARISON OF ABILITY OF Mg AND Mn TO ACTIVATE THE KEY ENZYMES OF GLYCOLYSIS

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1. Introduction

Mg and Mn are activating ions for all enzymes employing adenine nucleotides and for many others. Ca by contrast is often an inhibitor. Interaction between the ions has been suggested as a possible mechanism involved in the control of metabolism including the pathways of glycolysis and gluconeogenesis. We have already published some kinetic parameters for the interaction of these ions with the enzymes of hepatic gluconeogenesis [1]. The present study is concerned with the effects of bivalent ions on the activities of key glycolytic enzymes of liver.

2. Methods

All assays were performed at 30° and under optimal substrate conditions as determined in this laboratory. Fed rats were killed by decapitation, exsanguinated and the livers excised and frozen at −30°. Enzymic activity is expressed as μmole of substrate used or product formed per g wet liver per min.

Pyruvate kinase was assayed by a method based on that of Tanaka et al. [2]. Liver was homogenised in 9 vol of 0.15 M KCl containing 5 mM $MgSO_4$, 10 mM mercaptoethanol, 5 mM EDTA and 2 mg/ml bovine plasma albumin, final pH 7.3. The assay medium contained, in a final volume of 1 ml, 50 mM Tris-HCl pH 7.5, 5 mM $MgSO_4$ or 1 mM $MnCl_2$, 100 mM KCl, 0.2 mM NADH, 5 mM PEP, 2 mM ADP and 2 units lactic dehydrogenase. The reaction was started by the addition of 10 μl of the 100,000 *g* supernate diluted 1 to 4 and the disappearance of NADH followed spectrophotometrically.

Phosphofructokinase activity was measured by the method of Brock [3]. Liver was homogenised in 3 vol of 0.1 M K_2HPO_4 containing 10 mM mercaptoethanol. The pH was adjusted to 8.2 and the extract centrifuged at 38,000 *g* for 20 min. The assay medium contained, in a final volume of 1 ml, 50 mM Tris-HCl pH 8, 6.7 mM $MgSO_4$, 100 mM KCl, 0.3 mM KCN, 2 mM fructose 6-phosphate, 2 mM ATP, 2 mM dithiothreitol, 0.1 mM NADH, 5 μg triose phosphate isomerase, 5 μg α-glycerophosphate dehydrogenase and 80 μg aldolase. The rate of disappearance of NADH on addition of 10 μl of extract was followed.

Glucokinase and hexokinase were assayed in the same extract as pyruvate kinase by the method of Walker and Holland [4]. The medium contained, in a final volume of 1 ml, 50 mM Tris-HCl pH 7.5, 7.5 mM $MgSO_4$, 0.5 mM NADP, 5 mM ATP, 0.2 units glucose 6-phosphate dehydrogenase and 100 or 0.5 mM glucose. The reaction was initiated by the addition of 10 μl of the 100,000 *g* supernate and the reduction of NADP followed. Glucokinase activity was taken as the difference between the rate of NADPH formation in 100 and 0.5 mM glucose in the presence of 5 mM ATP, whilst hexokinase was defined as the activity with 0.5 mM glucose in the presence and absence of 5 mM ATP. Addition of glucose 6-phosphate to the assay medium led to reduction of one mole of NADP per mole of glucose 6-phosphate.

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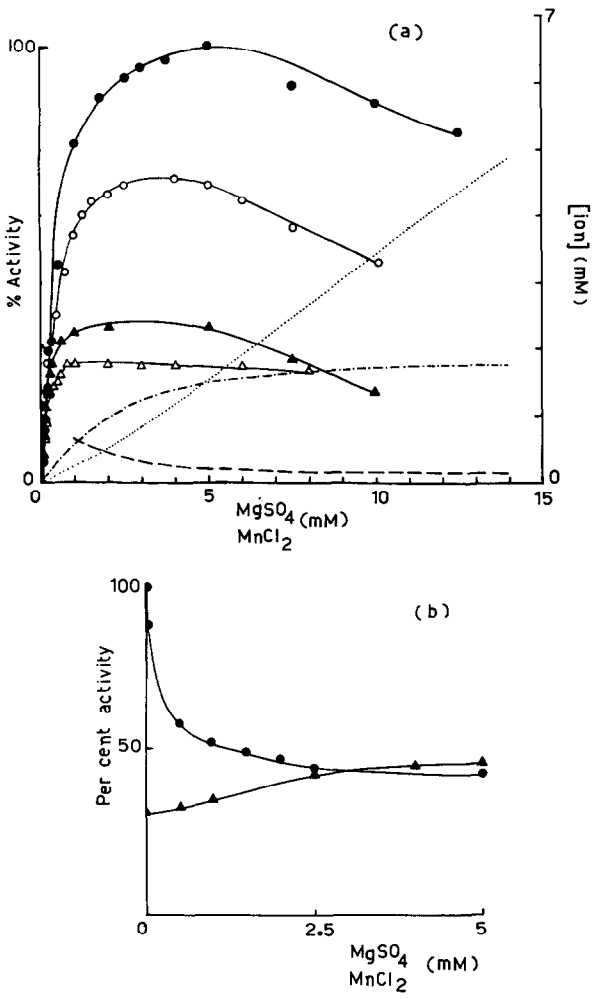

Fig. 1. Activation of pyruvate kinase by Mg or Mn. (a) ●—●—●, Activity in $MgSO_4$; ▲—▲—▲, activity in $MnCl_2$, · · · · · $[Mg^{2+}]$, – · – · – [Mg. ADP^{2-}], – – – $[ADP^{3-}]$, at 2 mM ADP.. ○—○—○, Activity in $MgSO_4$; △—△—△, activity in $MnCl_2$, at 0.05 mM ADP. (b) Activation of pyruvate kinase by both Mg and Mn. ▲—▲—▲, Increase of $MgSO_4$ with Mn held at 1 mM; ●—●—●, increase of $MnCl_2$ with $MgSO_4$ at 5 mM. Activity is expressed as a percentage of that under optimal assay conditions as described in Methods of 65 μmoles NADH oxidized/g liver/min.

Calculation of the relative concentrations of the various metal ion complexes were made by computer and took account of $[(NH_4)_2SO_4]$ added with the coupling enzymes in the assays. Stability constants assumed were (M^{-1}): $MgATP^{2-}$, 20,000; $KATP^{3-}$, 15; $MgADP^{-}$, 4,000; MgPEP, 180; $MgSO_4$, 180; $MgCl^{+}$, 3.5 [5–8].

3. Results

3.1. *Pyruvate kinase*

Unlike the rabbit muscle and ascites cell (type M) enzyme investigated by Bygrave [9], rat liver pyruvate kinase reached greater activity with Mg as the activating cation than with Mn (fig. 1a). Lowering the pH from 7.5 to 7 had no effect on the relative activation of the cations. High levels of either ion were inhibitory at concentrations of M^{2+} greater than $M.ADP^{-}$. The inhibitory effect of Mn in the presence of Mg was particularly pronounced (fig. 1b). Reciprocal plots of the data were nonlinear. Estimates of K_a were made from Hill plots and confirmed from Lineweaver–Burk plots of the form $1/v$ versus $1/s^n$ where n represents the degree of interaction of binding sites as ascertained from the Hill plot. K_a towards total Mg of 0.4 mM (n = 1.6) was half that for Mn (K_a 0.2 mM, n = 2). The value of n and a comparison of enzymic activity with the concentrations of the various ionic species shown in fig. 1a are consistent with both M^{2+} and $M.ADP^{-}$ being required by the enzyme. The decreasing activity at high levels of bivalent cations was unlikely to be due to increasing ionic strength– based on the calculated concentrations of the various ion species, ionic strength rose from 0.137 at 5 mM $MgSO_4$ to 0.149 at 11 mM $MgSO_4$. Over this range the activity of the enzyme decreased 20% and the [free Mg^{2+}] more than doubled (fig. 1a). Lowering ADP concentration to 0.5 mM makes the inhibition by Mg^{2+} more apparent, and shifts optimal activity in either cation to lower concentrations.

Addition of $MnCl_2$ to assays containing optimal amounts of $MgSO_4$ (5 mM) decreased the activity of the enzyme, 1.5 mM $MnCl_2$ producing 50% inhibition. Increasing concentrations of $MgSO_4$ in assays containing 1 mM $MnCl_2$ (slightly below optimal concentration) resulted in a 10% stimulation of activity (fig. 1b).

In common with pyruvate kinase from other sources [9] the rat liver enzyme was subject to inhibition by calcium ions. The inhibition was competitive with respect to either activating cation, but K_i in the two systems showed a 5-fold difference, i.e. 0.05 mM in the Mg activated assay as opposed to 0.25 mM in the Mn system (fig. 2).

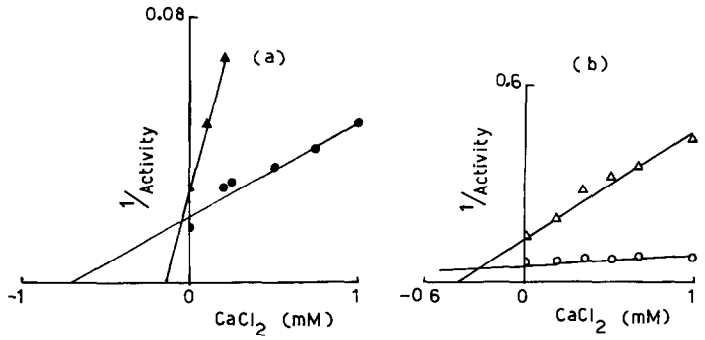

Fig. 2. Dixon plot of the inhibition of pyruvate kinase by Ca^{2+} assayed in (a) ●—●—●, 5 mM $MgSO_4$; ▲—▲—▲, 1 mM $MgSO_4$, (b) ○—○—○, 1 mM $MnCl_2$; △—△—△, 0.2 mM $MnCl_2$.

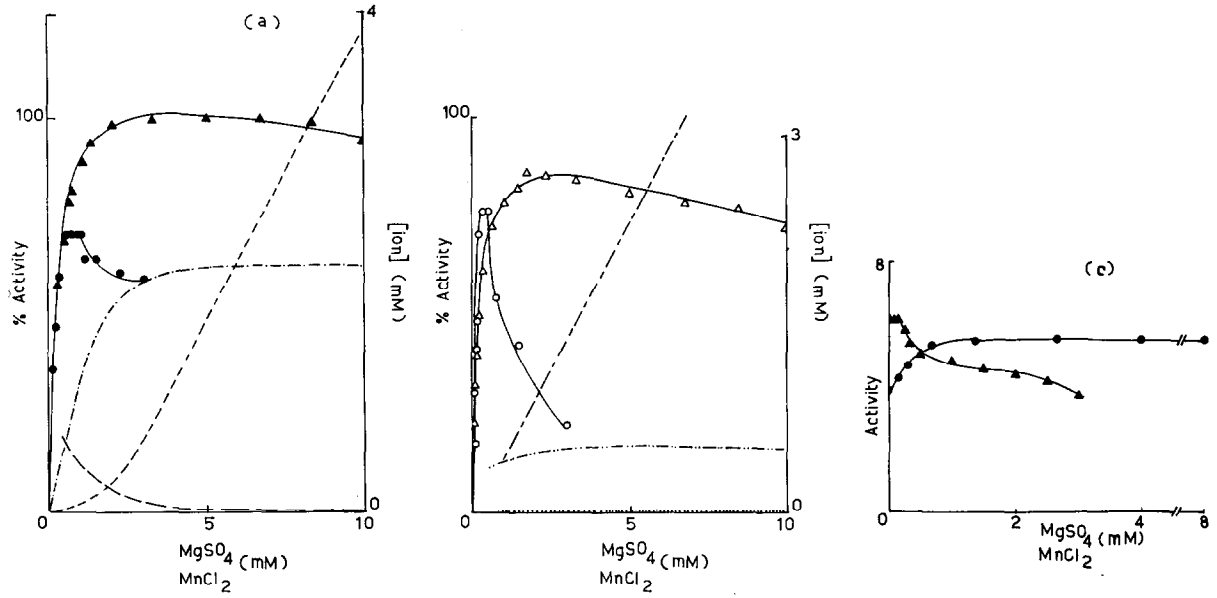

Fig. 3. Activation of phosphofructokinase by Mg or Mn. (a) ▲—▲—▲, Activity in $MgSO_4$; ●—●—●, activity in $MnCl_2$. - - - $[Mg^{2+}]$, – . – . – [Mg. ATP^{2-}], – – – $[ATP^{4-}]$, at 2 mM ATP. (b) △—△—△, Activity in $MgSO_4$; ○—○—○, activity in $MnCl_2$; – – – $[Mg^{2+}]$, – . . – [Mg. ATP^{2-}], · · · · $[ATP^{4-}]$, at 0.5 mM ATP. (c) Activation by both Mn and Mg. ▲—▲—▲, Increase of $MnCl_2$ with $MgSO_4$ at 6.7 mM; ●—●—●, increase of $MgSO_4$ with $MnCl_2$ at 0.5 mM. Activity in conditions as described in Methods is 6.6 μmoles NADH oxidised/g liver/min.

3.2. *Phosphofructokinase*

Phosphofructokinase was assayed in the presence of three activators, viz. NH_4^+, P_i and AMP. The last also prevents the recycling of FDP by fructose 1:6-diphosphatase, a possible contaminant of the relatively crude extract [10]. Under these conditions Mg and Mn both activated the enzyme, Mg producing higher activities than Mn at both pH 8 (fig. 3) and at pH 7.4. Although Mg activation showed similar features at both pH 7.4 and 8, with activity rising rapidly until Mg was equimolar with ATP, the apparent K_a was slightly different, being 0.43 mM at pH 7.4 and 0.25 mM at pH 8. (For

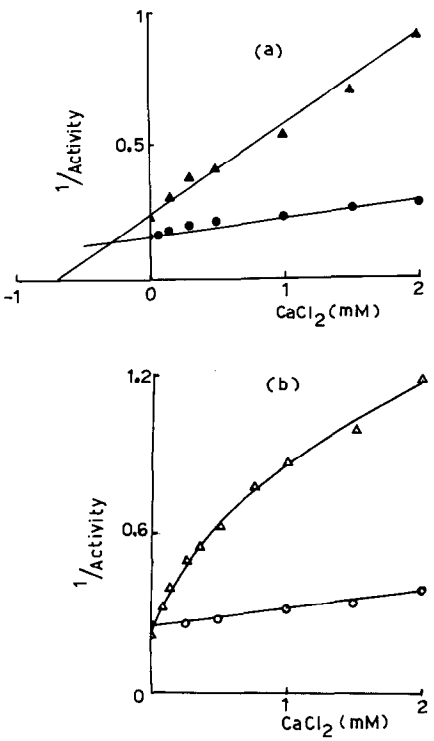

Fig. 4. Dixon plot of the inhibition of phosphofructokinase by Ca^{2+} assayed in (a) ●—●—●, 6.7 mM $MgSO_4$; ▲—▲—▲, 0.5 mM $MgSO_4$; (b) ○—○—○, 2 mM $MnCl_2$; △—△—△, 0.5 mM $MnCl_2$.

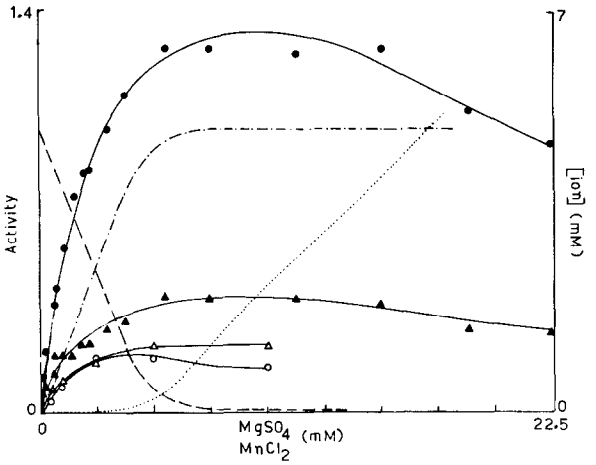

Fig. 5. Activation of glucokinase and hexokinase by bivalent cations. ●—●—●, Glucokinase activity with $MgSO_4$; ○—○—○, glucokinase activity with $MnCl_2$; ▲—▲—▲, hexokinase activity with $MgSO_4$; △—△—△ hexokinase activity with $MnCl_2$. · · · · [Mg^{2+}], – · – [Mg. ATP^{2-}], – – – [ATP^{4-}], at 5 mM ATP. Activity units: μmoles NADPH formed/g liver/min.

comparison, K_m towards ATP at the latter pH was 0.13 mM.) Apparent K_a towards Mn was the same at both pH at 0.16 mM. At each of these concentrations of activator, M^{2+} is less than 0.05 mM, whilst M.ATP^{2-} is in the range 0.1–0.4 mM. With the exception of Mg dependence at pH 8, n, derived from Hill plots, was 1.4 with respect to total [ion]; at pH 8 in the Mg activated assay, n was 1.2. Lowering [ATP] does not affect the apparent K_a for either total Mg or Mn.

Reduction of the concentration of ATP to 0.5 mM enhanced inhibition of the enzyme by the cations (fig. 3b). If, as suggested for the rabbit muscle enzyme [11], free metal ions are essential for activity then the affinity of phosphofructokinase for M^{2+} must be high, since even with Mg optimal activity was approached when the total Mg level was equal to that of ATP. At these concentrations free Mg^{2+} was 0.2 mM, compared with $MgATP^{2-}$ 1.6 mM and ATP^{4-} 0.15 mM.

Fig. 3c demonstrates the effects at pH 8 of combinations of metal ions: increasing [$MnCl_2$] at optimal [Mg] reduced activity to that in the absence of Mg, but addition of $MgSO_4$ at 0.5 mM $MnCl_2$ produced a stimulation of activity to a point intermediate between those found with either ion alone.

Ca, as with pyruvate kinase, inhibited phosphofructokinase by competition with Mg (fig. 4a), K_i being about 0.22 mM. Inhibition with respect to Mn was more complex (fig. 4b), with n as calculated from Hill plots rising from 0.65 at 0.5 mM $MnCl_2$ to 1.45 at 2 mM $MnCl_2$. At both concentrations of Mg employed, n for Ca was 0.85.

3.3. *Hexokinase and glucokinase*

Fig. 5 shows the Mg requirement of the two enzymes assayed in 5 mM and 1 mM ATP. Maximal activity was reached for both at a Mg:ATP ratio of 1:1, with loss of activity when this ratio exceeded 3:1. A similar situation is reported for rat muscle hexokinase [12]. Calculation of the levels of the ion species present showed that the inhibition became significant as [Mg^{2+}] exceeded [$MgATP^{2-}$]. Half maximal activation of hexokinase by$MgSO_4$ with ATP at 5 mM was reached at 1.1

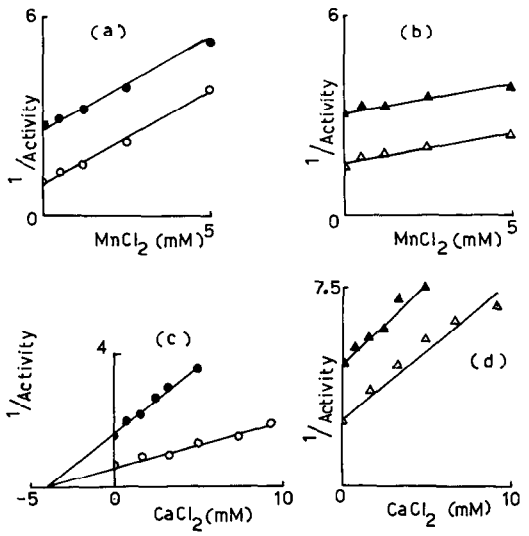

Fig. 6. Dixon plots of the inhibition of glucokinase and hexokinase by Mn^{2+} and Ca^{2+}. (a) Inhibition of glucokinase by $MnCl_2$ in ○—○—○, 7.5 mM $MgSO_4$; ●—●—●, 1.13 mM $MgSO_4$. (b) Inhibition of hexokinase by $MnCl_2$ in △—△—△, 7.5 mM $MgSO_4$; ▲—▲—▲, 1.13 mM $MgSO_4$. (c) Inhibition of glucokinase by $CaCl_2$ in ○—○—○, 7.5 mM $MgSO_4$; ●—●—●, 1.13 mM $MgSO_4$. (d) Inhibition of hexokinase by $CaCl_2$ in △—△—△, 7.5 mM $MgSO_4$; ▲—▲—▲, 1.13 mM $MgSO_4$.

mM and for glucokinase at 1.13 mM. At these concentrations the predominant form of Mg is $MgATP^{2-}$, with Mg^{2+} less than 10 μM and therefore unlikely to play a significant role in the activation of the enzyme. There was a marked difference in the responses of the two enzymes to activation by Mn by comparison with Mg. Glucokinase was virtually inactive (fig. 5), but hexokinase showed half the activity found in the Mg assay and had a K_a of 1 mM.

Mn was an uncompetitive inhibitor of both hexokinase and glucokinase (fig. 6a, b) with respect to Mg. Glucokinase was more sensitive than hexokinase to this inhibition, with 50% loss of activity caused by 2 mM $MnCl_2$ in the presence of 7.5 mM $MgSO_4$; a similar fall in hexokinase activity required 5 mM $MnCl_2$. Whereas n from Hill plots was 1 for Mn binding to the former enzyme, for hexokinase n was 0.6 at both Mg levels studied. Calcium also inhibited the enzymes, noncompetitively with respect to Mg for glucokinase (K_i 4 mM), but tending to uncompetitive for hexokinase (fig. 6c, d). 50% inhibition however occurred at similar levels for both enzymes between 4 and 5 mM $CaCl_2$ in the presence of 7.5 mM $MgSO_4$.

4. Discussion

The present results add to the general picture that many, though not all, enzymes requiring Mg can also be activated by Mn. In general the optimal concentration of Mn is lower than for Mg. The reason for this is not known. Hexokinase is an exception to this in that the K_a for the two metal ions is similar – possibly because in this case the requirement of the bivalent ion is solely related to its chelation by ATP without there being evidence of interaction of the metal ions with the enzyme [13]. That glucokinase should under these circumstances not respond to Mn is surprising.

Detailed analyses have been made of the role of bivalent cations in the activation of type M pyruvate kinase from a variety of sources [9, 14, 15], but relatively little data is available for the hepatic enzyme which has different substrate affinity and activator requirements from the muscle form [16]. Mildvan and Cohn [17] showed direct binding of Mn and Mg to the active site of the rabbit muscle enzyme with competitive inhibition by Ca ions, the number of interacting sites (n) being equal to two. Kayne and Seulter [14] however suggested that the binding of the cations determined the enzyme conformation rather than being at the active site. We have found similar multiple binding of Mn or Mg with pyruvate kinase from rat liver, with the further suggestion that free Mn^{2+} in excess of $M.ADP^{2-}$ is inhibitory.

Friedmann and Rasmussen [18] have shown that low concentrations of $MnCl_2$ stimulate gluconeogenesis by the perfused rat liver, without altering [cyclic AMP]. Addition of Ca had no effect (by comparison with a stimulatory effect in kidney slices [19]), though the synthesis of glucose was accompanied by efflux of Ca from the liver. Comparison of the cation sensitivity of the rate limiting enzymes of glycolysis and gluconeogenesis shows phosphoenolpyruvate carboxykinase to have the greatest sensitivity to Mn, 10 μM $MnCl_2$ activating the enzyme *in vitro* several fold [1, 20]. This activation of the soluble enzyme is not related to a change in its affinity for its substrate oxaloacetate for, although the K_m of the mitochondrial enzyme is lower in the presence of Mn as opposed to Mg, that of the

cytosolic enzyme is unaltered [21]. Activation of fructose 1:6-diphosphatase in the absence of Mg also occurs at lower [Mn] than those able to modify the activity of the glycolytic enzymes, but if Mg is present these same [Mn] become inhibitory.

Movement of Mn across the mitochondrial membrane is accompanied by a similar redistribution of Ca [22]; thus cytosolic Mn and Ca alter together. Of the glycolytic and gluconeogenic enzymes, pyruvate kinase is the most sensitive to Ca by a factor of 3. Any role of bivalent cations in the control of glycolysis and gluconeogenesis is likely to concern the relative concentration of all three, increased cytosolic levels of Mn and Ca probably favouring the synthesis of glucose in the liver.

Acknowledgement

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